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TITLE: Reversal of Estrogen Receptor Beta Epigenetic Gene Silencing in Prostatic Adenocarcinoma by Soy Protein-Derived Isoflavonoid Supplementation

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14. ABSTRACT Purpose: The purpose of this study was to evaluate the effect of a 4-week soy isoflavonoid treatment at a dose of 100mg/day on DNA promoter methylation density of the estrogen receptor β (ER β) gene in men with organ localized prostate cancer. We hypothesized that ER epigenetic silencing through DNA promoter methylation in neoplastic prostatic epithelial cells can be reversed by dietary soy isoflavonoids when given at physiologically achievable levels, as shown by a positive correlation between serum and/or intraprostatic isoflavonoid concentrations and ER β expression and an inverse correlation with neoplastic cell proliferation. Results: A 4-week treatment with soy isoflavonoids did not result in a decrease in ER β DNA promoter methylation within the prostate tissue. ER β protein expression did not differ when analyzed by treatment group in prostatic adenocarcinoma, normal prostate tissue, or BPH. Isoflavonoid concentrations within the prostate tissue did not concentrate above serum levels in the soy isoflavonoid treatment group. ER α DNA promoter methylation in prostatic adenocarcinoma was increased in the soy treatment group when compared to the placebo protein treatment group (P<0.02). A concomitant decrease in ER α protein expression was seen in the neoplastic prostate (P<0.06) epithelium. Conclusion: Short-term soy isoflavonoid at 100mg/day did not alter estrogen receptor β DNA promoter methylation but increased ER α DNA promoter methylation in the neoplastic prostate tissue.					
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Introduction

Prostate cancer is the most common noncutaneous cancer in men and the second leading cause of cancer death with 186,320 estimated new cases diagnosed in 2008.¹ African American men have the highest prostate cancer incidence, being 1.5 times more likely to develop the disease when compared to Caucasian American men.² Prostate cancer incidence in Asian American men is even lower being one- to threefold lower than that seen in Caucasian American men.^{3,4} This gap in incidence is more pronounced if the incidence rate is compared to that of Asian men living in their country of origin, where the incidence is ten to sixty times lower than the incidence in African American and Caucasian American men living in the United States, although prostate cancer incidence is increasing yearly in all races.⁵⁻⁷ (**Figure 1**)

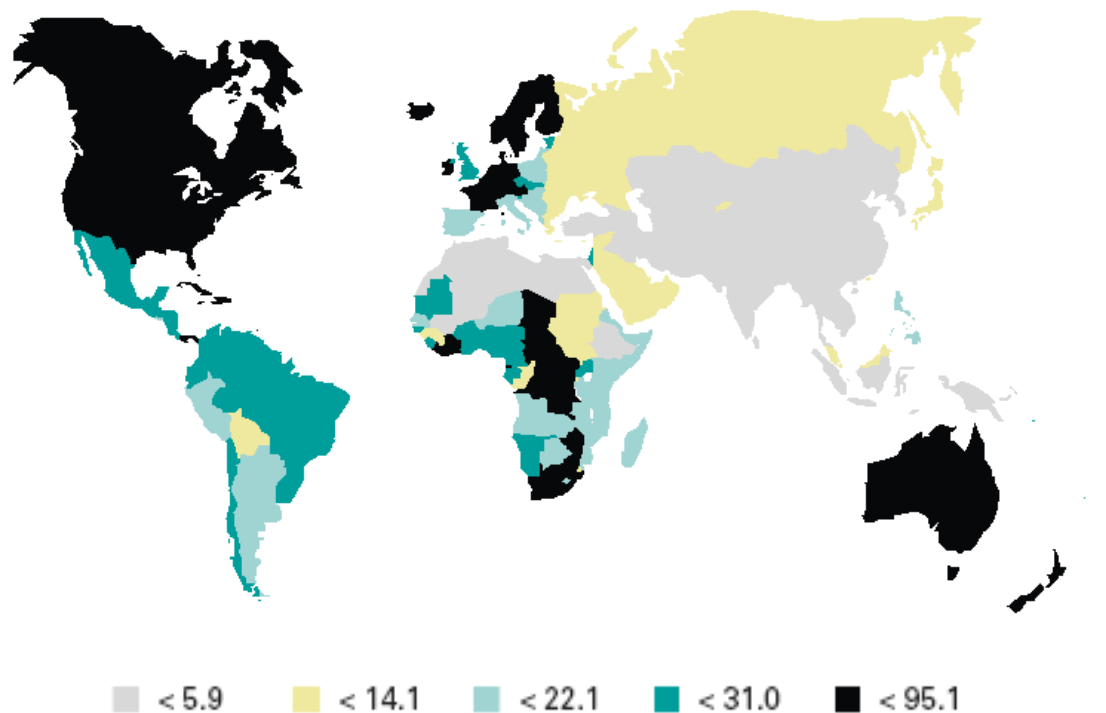


Figure 1. The incidence of prostate cancer world-wide (per 100,000 individuals), age-standardized using the World standard population. GLOBOCAN (IARC 1998) Quinn et al. *British Journal of Urology International* 2002.

Disparate socioeconomic status, diagnostic screening, serum and tissue hormone levels, prostate tissue sex steroid receptor expression levels, diet, obesity, and gene expression have all been proposed to explain the racial discrepancies in prostate cancer incidence seen in African and Caucasian American men when compared to men of Asian descent.^{2,8-27}

Interestingly, the incidence of a controversial precursor lesion, prostatic intraepithelial neoplasia,²⁸ follows a parallel epidemiologic pattern.²⁹ In men without concurrent prostate cancer, African American men have twice the frequency of prostatic intraepithelial neoplasia, than Caucasian men of similar age,³⁰ and multifocal to diffuse high grade prostatic epithelial neoplasia is detected a decade earlier in the prostate glands of African American men (men in their 20s)³¹ than in either Caucasian (**Figure 2**) or Asian men although this is based on a single, widely-cited, study.³²

In contrast to men without prostate cancer, men with prostate cancer have a similar incidence of concurrent prostatic intraepithelial neoplasia regardless of race, ranging from 76% to 86%.^{29,31,33-35} It is widely recognized that African and Caucasian American men develop more clinically apparent tumors with higher mortality than Asian men.^{6,22,36} In addition, African American men typically develop clinically relevant disease 5 years younger than Caucasian men.³⁷ Thus, these recorded incidence rates of prostatic intraepithelial neoplasia appear to parallel the racial discrepancy in prostate cancer, and further studies into the role of prostatic intraepithelial neoplasia in the progression to malignancy are needed.

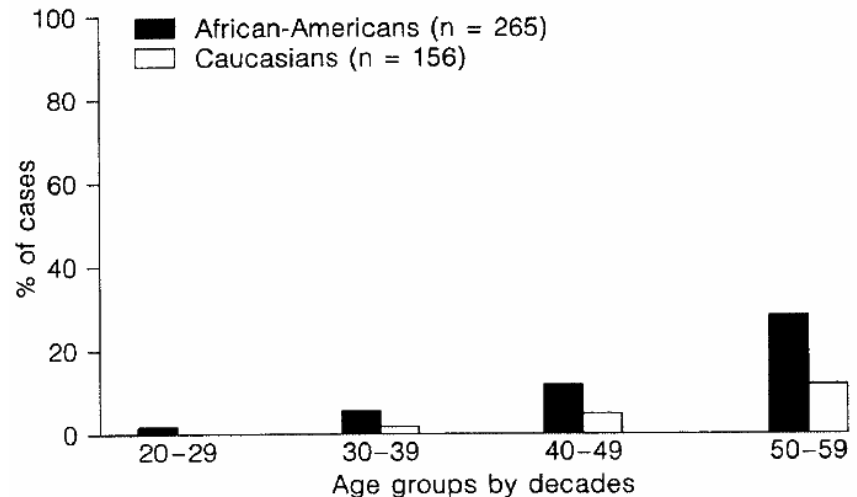


Figure 2. High grade prostatic intraepithelial neoplasia with diffuse involvement of the gland appears at a younger age in African American men than in Caucasian men. Autopsy study. Sakr et al. *European Journal of Urology* 1996.

Androgens are required for normal prostate growth and maintenance.³⁸ Testosterone and dihydrotestosterone are mitogenic in the prostate gland, stimulating prostate epithelial and stromal cell growth, differentiation, and maintenance through androgen receptor binding and transactivation.³⁹ Castration before the onset of puberty, depriving the body of androgen production by testicular leydig cells, prevents the prostate gland from developing.^{40,41} A widely held hypothesis was that circulating and intraprostatic androgen concentrations would, by acting as powerful mitogens, parallel the race disparities seen in prostatic intraepithelial neoplasia and prostate cancer, thereby explaining the marked disparities seen in prostate cancer incidence rates by race.

Unfortunately, the role of androgens in prostate cancer development is not that clear cut. While many early studies reported testosterone and dihydrotestosterone concentrations or the ratio of dihydrotestosterone to testosterone to be higher in African American men than in Caucasian men and even lower circulating concentrations in Asian men,^{38,42-44} the majority of more recent publications have found no significant differences in androgen concentrations in men with prostate cancer when examined by race and no significant increased risk of developing prostate cancer with higher circulating androgen concentrations.^{11,23,45-51} In fact, some studies have reported lower testosterone levels are associated with an increased incidence of prostate cancer and/or higher prostate tumor grade,⁵²⁻⁵⁷ and some publications report no differences or association between circulating or intra-tissue androgen or estrogen levels with increasing prostate cancer grade.^{11,12} Thus, the role of androgen exposure in prostate cancer development remains unclear. It has also been suggested that significant differences in circulating hormone levels that might lead to increased prostate cancer risk may be detectable only in young men under the age of 35, before the onset of andropause,^{42,58} or differences in *in utero* exposure to circulating hormones in the mothers of men that develop prostate cancer⁵⁹ may be the predisposing factor leading to an increased risk for prostate cancer. From an epigenetic standpoint, both are intriguing possibilities.

Androgen receptor protein expression is maintained even in the face of androgen-independent prostate cancer.⁶⁰ Increased androgen receptor expression has been reported in the neoplastic prostate epithelium of African American men when compared to Caucasian men^{24,61} although the impact on disease progression of increased or decreased androgen receptor expression levels in neoplastic prostate tissue is controversial. Prostate tumors exhibiting more androgen receptor expression have been associated with more aggressive disease⁶² while others have found expression levels not to be a useful prognostic indicator^{63,64} and have reported lower androgen receptor expression in neoplastic prostate epithelium when compared to expression levels in normal prostate epithelium.^{62,64} In the supporting stroma of the prostate gland, lower androgen receptor expression has been associated with more aggressive disease.⁶⁵

Common androgen receptor microsatellite trinucleotide repeats include CAG, encoding polyglutamine, and GGN, encoding polyglycine, both located within the NH₂-terminal (transactivation) domain of the androgen receptor protein.⁶⁶ Shorter polyglutamine tracts (shorter CAG repeats) *in vitro* are more transcriptionally active, and in men, shorter CAG repeats have been associated with more aggressive prostate tumors and diagnosis at a younger age.⁶⁶ This description of earlier age at diagnosis with higher prostate cancer grade is consistent with the epidemiologic data of prostate cancer incidence in African American men. Indeed African American men have been reported to have the shortest polyglutamine tracts (CAG repeats) in the transactivation domain of their androgen receptors,⁶⁷ while Caucasian men have intermediate length CAG repeats. Men of Asian descent have the longest CAG repeats, or the least transcriptionally active androgen receptors,⁶⁸ but more recent studies have demonstrated no association between CAG or GGN repeats and prostate cancer risk in African American men.^{69,70} Short GGN repeats are associated *in vitro* with hairpin formation of the mRNA transcript resulting in decreased mRNA translation of the androgen receptor transcript and presumably decreased androgen receptor protein expression in the prostate epithelium and stroma *in vivo*. A recent study in men with short GGN repeats has found no difference in androgen receptor staining immunohistochemically, but did find shorter GGN repeats were associated with higher Gleason score.⁷¹ The true clinical significance of these androgen receptor polymorphisms in prostate cancer risk remains unresolved.

Androgen receptor mutations that have been reported in prostate cancer are numerous (approximately 60), but the incidence is generally reported to be low, detected in less than 5% of prostate tumors.⁷² A shared feature of these mutations is either enhanced ligand response to androgen and/or ligand promiscuity. These mutated receptors gain the ability to utilize adrenal-derived androgens, estrogens, and progesterone among other hormone or hormone-like molecules as ligands through mutations in the region of the ligand binding pocket. However, mutations in other regions of the protein also have been reported that may enhance androgen receptor stability, coactivator interactions, transactivation, and nuclear translocation, even in the absence of ligand binding. Unfortunately, androgen deprivation therapy appears to place selection pressure on prostate tumor cells, as these cells have more mutations in the ligand binding domain of their androgen receptors than those of untreated men,⁷² and androgen deprivation therapy may promote the development of androgen receptors that lack a ligand binding domain entirely, yet retain the ability to induce androgen receptor signaling.⁷³

Clearly additional research into the role of androgen hormones and the androgen receptor in prostate cancer development and progression is necessary, although we have learned that while androgen hormones and their receptors are necessary for prostate gland development, they alone do not lead to the development of prostate cancer.

Another salient difference between American men and Asian men is their diet. A traditional Asian diet is rich in soy protein⁷⁴ and lower in saturated fat than an American diet.¹⁵ North American, European, and Australian men consume 1-2g (3-6mg isoflavonoids) of soy protein daily, while Asian men consume an estimated 8-11g (24-30mg isoflavonoids) daily.⁷⁴ Interestingly, prostate cancer incidence doubles in Asian American immigrants between the ages of 45-69 with consumption of a Western diet, although the prostate cancer incidence in Asian Americans remains approximately half that seen in Caucasian American men,^{6,26,75,76} indicating that both genetic differences and diet likely play a role in prostate cancer development.

Studies designed to demonstrate a decrease in prostate cancer risk with consumption of higher concentrations of soy isoflavonoids have demonstrated mixed results.⁷⁷ Some studies conclude no effect of soy consumption on prostate cancer risk,^{16,78} while other studies demonstrate a decreased risk of prostate cancer in men that consume higher concentrations of soy.⁷⁹⁻⁸⁴ These studies vary widely in design, sample size, patient age, types of soy-containing foods included in dietary questionnaires and/or type of soy food consumed, time course, and in primary outcome (tumor of any grade detected, rise or fall in PSA value). However, the epidemiologic evidence for a role of soy consumption in reducing prostate cancer risk remains compelling and consistent.

Soy protein contains estrogen-like compounds, termed isoflavonoids. Isoflavonoids are polyphenolic compounds that are structurally similar to the endogenous sex steroid hormone estradiol (17 β estradiol) and are able to bind to the estrogen receptor with varying affinities^{85,86} and may have agonistic or antagonistic effects⁸⁷ depending on species, dose, cell type, ratio of estrogen receptor subtype expression in the tissue, and hormone milieu.^{88,89} In the prostate gland both estrogen receptor subtypes are expressed, although estrogen receptor α expression predominates in the stroma, and estrogen receptor β is expressed predominantly in the epithelium.^{90,91} The effects of estrogen or estrogenic compounds in the prostate are mediated through these two estrogen receptors but they are thought to have opposing effects. Estrogen receptor α promotes proliferation, squamous metaplasia, and inflammation or prostatitis, and estrogen receptor β activation has antiproliferative and anti-inflammatory effects.^{90,92} The antiproliferative effect of estrogen receptor β has been demonstrated in cell culture studies^{93,94} and in a variety of murine animal models since 1998.^{95,96}

Epithelial hyperplasia of the prostate gland has been reported in estrogen receptor β knockout mice.⁹⁴ Prins et al. provided additional insight into the role of the two estrogen receptor subtypes in a study of neonatal estrogen imprinting in 2001. In this study, wild-type mice treated from days 1 through 5 of life with diethylstilbestrol (2 μ g/day) exhibited expansion of the stromal compartment of the prostate gland, a continuous layer of basal cells (mice normally have a discontinuous basal cell layer lining prostatic acini), periductal fibrosis, lymphocytic prostatitis, and epithelial hyperplasia and dysplasia that increased in severity with age. These changes are termed “developmental estrogenization.” Estrogen receptor α knockout mice (retain estrogen receptor β expression) treated with the same dose of diethylstilbestrol (DES) for 5 days exhibited none of the pathologic changes exhibited by the wild-type mice, while estrogen receptor β knockout mice (retain estrogen receptor α expression) exhibited the same pathologic changes seen in wild-type mice: expansion of the stromal compartment, lymphocytic prostatitis, and epithelial hyperplasia and dysplasia in addition to a continuous basal cell layer lining prostatic acini and periductular fibrosis. These estrogen receptor β knockout mice also exhibited decreased androgen receptor in the dorsolateral lobe of the prostate as adults when compared to estrogen receptor α knockout mice. These findings indicate that estrogen receptor α mediates neonatal imprinting or “developmental estrogenization” of the prostate gland.⁹⁷

McPherson et al. demonstrated a similar antiproliferative role for estrogen receptor β in the prostate gland using aromatase knockout mice. In the absence of stromal and epithelial

aromatase, local estrogen production in the prostate gland is absent. These animals exhibit epithelial hyperplasia that can be abrogated by treatment with an estrogen receptor β agonist. Estrogen receptor β agonist treatment in these mice made their prostate gland indistinguishable from wild-type controls. Exposure of the same aromatase knockout animals to an estrogen receptor α agonist did not abrogate the prostatic epithelial hyperplasia and resulted in an increased inflammatory response within the gland.⁹⁸

Savolainen et al. utilized luteinizing hormone knockout (LuRKO) mice to elucidate the role of the two different estrogen receptors in the prostate gland. These mice lack postnatal androgen production in the testes because they lack pituitary gland luteinizing hormone release/regulation of the hypothalamic-pituitary-gonadal axis. These mice were given exogenous testosterone or dihydrotestosterone replacement to facilitate normal prostate gland development for 8 or 16 weeks and a variety of hormone treatments. In testosterone propionate-treated male LuRKO mice, prostatic epithelial acinar hyperplasia was reversed by treatment with the estrogen receptor beta agonist DPN (2, 3-bis(4-hydroxyphenyl)-propionitrile), indicating again that activation of the estrogen receptor β subtype has antiproliferative effects in the prostate gland epithelium.⁹⁹

Expression of estrogen receptor β in organ-localized neoplastic prostate cancer in men is reported to decrease with neoplastic transformation in the majority of publications,^{91,100-104} although some report increases in estrogen receptor β expression with neoplastic transformation.^{105,106} In normal and neoplastic prostate tissue, expression of the two estrogen receptor subtypes is controlled mainly through DNA promoter methylation.¹⁰⁷⁻¹⁰⁹ In prostate cancer cell lines treated with demethylating agents and histone deacetylating agents, increases in estrogen receptor β expression resulted in decreased proliferation and increased apoptosis.¹⁰⁸ Dietary isoflavonoids have been shown to alter DNA promoter methylation in the prostate gland of mice,¹¹⁰ and to reverse DNA promoter methylation of a variety of genes in human prostate cancer cell lines, including tumor suppressor genes¹¹¹ such as retinoic acid receptor β , a similar nuclear steroid receptor to the estrogen receptor in the human prostate cancer cell line PC-3.¹¹²

In light of the epidemiologic evidence, it is likely that variations in prostate cancer incidence within different populations and ethnicities are due to a combined genetic-nutrient interaction.^{15,19,113,114} The goal of this project is to study such a genetic-nutrient interaction using prostate tissue collected from 62 men with prostate cancer who voluntarily enrolled in a clinical trial (NIH R03 CA 103111, JM Cline) designed to evaluate the effects of soy protein consumption on the prostate. These men were given 50g of soy protein, containing 100 mg isoflavonoids (in the form of two 25g soy protein “shakes” daily) or milk protein daily for 4 weeks prior to radical prostatectomy.

We hypothesized that:

Estrogen receptor β (ER β) epigenetic silencing through DNA promoter methylation in neoplastic prostatic epithelial cells can be reversed by dietary soy isoflavonoids when given at physiologically achievable levels, as shown by a positive correlation between serum and/or intraprostatic isoflavonoid concentrations and ER β expression and an inverse correlation with neoplastic cell proliferation.

Body

Research Strategy:

Specific Aim 1: To determine intra-prostatic tissue soy isoflavonoid concentrations and compare these concentrations to those measured in serum.

Hypothesis: Soy isoflavonoids concentrate within prostatic tissue at levels 4-8 fold above those found in serum.

Specific Aim 2: To determine ER β DNA promoter CpG island methylation within prostate tissue collected from men treated with soy protein containing isoflavonoids and men receiving casein lactalbumin animal derived protein.

Hypothesis: ER β DNA promoter CpG island methylation will be decreased within prostatic tissue in men treated with soy protein containing isoflavonoids when compared to those receiving a similar concentration of animal derived protein.

Specific Aim 3: Correlate intraprostatic soy isoflavonoid concentrations and ER β DNA promoter methylation data with ER β protein expression data and immunohistochemical markers for cell proliferation.

Hypothesis: Soy isoflavonoids will concentrate in prostatic fluid resulting in a reduction in ER β DNA promoter methylation decreasing prostate cancer cell proliferation and increasing apoptosis.

Experimental design:

Following written consent, men diagnosed with prostatic adenocarcinoma by Ralph Woodruff, MD, a board-certified pathologist, were enrolled in a phase IIb clinical trial (NIH R03 CA 103111, JM Cline) and given 60 protein powder packets containing either soy protein or a control protein powder containing casein lactalbumin (milk protein-derived). These men were instructed to consume one 25g protein powder packet mixed in their beverage of choice twice daily for four weeks, between the time of biopsy diagnosis of prostatic adenocarcinoma and radical prostatectomy. Fifty grams of soy protein provided 100mg isoflavonoids per day

Nutritional Information:		
Serving Size:	36.5g	
Amount per serving:		
Calories:	130	
Calories from fat:	5-10	
		% Daily Value
Total Fat:	0.5-1g	1-2%
Saturated Fat:	0g	0%
Cholesterol:	0-5mg	0-2%
Sodium:	150-240mg	6-10%
Potassium:	10-200mg	0-6%
Total Carbohydrate:	5-6g	2%
Dietary Fiber:	0g	0%
Sugars:	3g	
Protein:	25g	50%
Protein from SUPRO® SOY™:	25g	
		%RDI
Vitamin A:	625IU	10%
Calcium:	900mg	90%
Vitamin D:	125IU	30%
Folate:	37.5mcg	8-20%
Phosphorus:	525mg	60%
Vitamin C	0mg	0%
Iron	1-4mg	5-20%
Riboflavin	0.5mg	30%
Vitamin B12	1mcg	15%
Magnesium	50-140mg	10-35%
Ingredients: Isolated soy protein or mild protein isolate; malodextrin, sucrose, fructose, artificial flavor. Vitamins and minerals: Calcium phosphate, magnesium phosphate, riboflavin, vitamin A palmitate, folic acid, vitamin D3, vitamin B12.		
SUPRO® SOY™ Proteins are part of a family of products represented by Solae™ brand soy protein. Placebo product produced with mild protein isolate		
Note Placebo product produced with milk protein isolate		

Table 1. Nutritional information for the soy protein and placebo (milk) protein powder.

to men randomized to the soy protein treatment group. (**Table 1, previous page**) Sixty-two men were randomized into the study. These men did not differ significantly in signalment, prostate specific antigen level (PSA), or biopsy and surgical tissue Gleason score. (**Table 2**)

Patient & Tumor Characteristics			
	Placebo (31) Milk Protein	Soy (31) Soy Protein	P
Age	59	58	0.4
Body Weight (lbs)	196	189	0.5
Height (inches)	70	70	0.8
Body Mass Index	28	27	0.3
Bx Gleason Sc	6	6	0.8
Sx Gleason Sc	6.5	6.5	0.9
Biopsy PSA (ng/mL)	5.9	5.2	0.6
Surgery PSA (ng/mL)	5.5	5.0	0.9

Table 2. Patient characteristics. Patient signalment and prostate gland tumor characteristics did not significantly differ when analyzed by treatment group.

Blood samples were taken before treatment and on the day of radical prostatectomy to evaluate pre-treatment and post-treatment serum isoflavonoid levels to allow comparisons between serum and intra-prostatic concentrations. (**Figure 3**)

Tissue collected at prostatectomy (by Dr. Perry) was stained with toluidine blue to identify epithelial cells, and was then evaluated under a

dissecting microscope to confirm that we were, in fact, obtaining foci of adenocarcinoma for snap freezing (these lesions are often multifocally distributed within the gland).¹¹⁵ Following rapid examination of the stained fresh tissue, five to seven 8mm punch biopsy sections were collected and snap frozen in liquid nitrogen for storage at -80°C. Two of these sections were from foci of adenocarcinoma, one immediately adjacent to adenocarcinoma and two to four areas from tissue that appeared grossly normal under the dissecting microscope. The

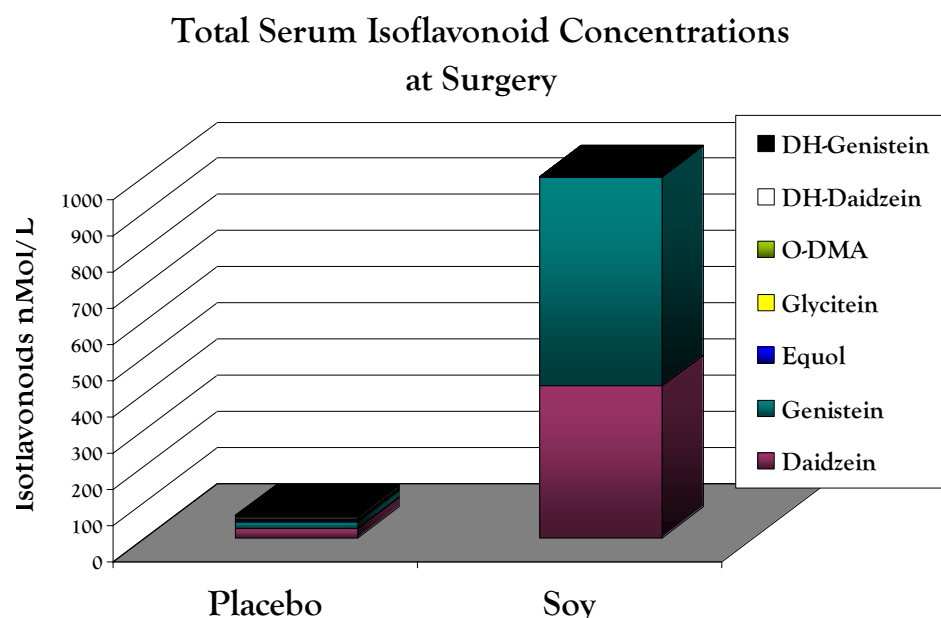


Figure 3. Serum soy isoflavonoid concentrations in placebo protein treated and soy protein treated men after 4 weeks of dietary treatment ($p < 0.01$).

remaining tissue was fixed in paraformaldehyde and paraffin embedded for routine histology and immunohistochemical analysis. Routine histology was used to confirm that frozen sections contained foci of adenocarcinoma. The fresh frozen, fixed prostate tissue we have collected was used to address our three specific aims.

Specific Aim 1: To determine intra-prostatic tissue soy isoflavonoid concentrations and compare these concentrations to those measured in serum.

In this aim we will examine whether or not isoflavonoids concentrate within the prostate glandular tissue, as it has been previously published that isoflavonoids concentrate at levels 4- to 23-fold above the serum in prostatic fluid.¹¹⁶⁻¹¹⁹

Dr. Adrian Franke from the Cancer Research Center of Hawaii performed liquid chromatography mass spectrometry on snap frozen sections (one 8mm punch biopsy) of prostate tissue taken at prostatectomy to assess intra-tissue isoflavonoid concentrations. Dr. Franke has extensive experience measuring concentrations of genistein, dihydrogenistein, daidzein, dihydrodaidzein, glycitein, and O-desmethylangolensin concentrations using liquid chromatography photodiode array mass spectrometry (LC-PDA-MS) assay in serum, as published previously.^{120,121} These intra-tissue concentrations were compared to those in the pre- and post-treatment serum samples from both the control and soy protein isoflavonoid treatment groups.

Results specific aim 1:

Isoflavonoid concentrations in the tissue were 33.4 nm/kg in the placebo protein treatment group and 98.8 nm/kg in the tissue of the soy protein treatment group, while post-treatment concentrations in the serum of the soy protein treatment group were 1077 nmol/L. Isoflavonoids measured in the tissue did not concentrate above the levels measured in the serum (**Figure 4**).

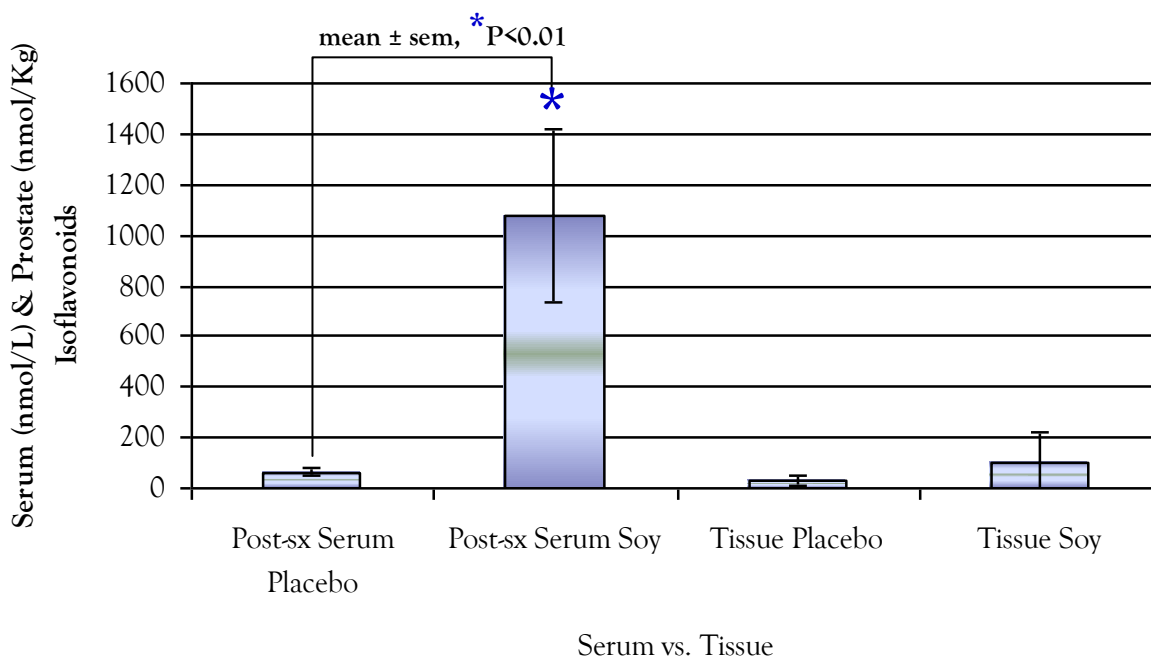


Figure 4. Serum Isoflavonoid Concentrations Post -Treatment vs. Intraprostatic Tissue Isoflavonoid Concentrations Post-Treatment. Isoflavonoids increased significantly in the serum with 4 weeks of isoflavonoid treatment ($P<0.01$) but did not concentrate in the prostate tissue at the time of tissue collection.

Aim 2: To determine ER β DNA promoter CpG island methylation within prostate tissue collected from men treated with soy protein containing isoflavonoids and men receiving casein lactalbumin animal-derived protein.

This aim will examine if a) ER β DNA promoter CpG island methylation occurs in neoplastic epithelial cells within the prostate gland, and b) if dietary treatment with soy protein-derived isoflavonoids can reverse ER β promoter methylation.

DNA methylation status within the promoter region of the ER β gene was assessed in both the control protein and soy protein-treatment groups following DNA isolation from snap frozen prostate tissue. Ten to twenty 10 μ paraffin sections of formalin-fixed prostate tissue were examined under a dissecting microscope. Neoplastic epithelial cells, normal epithelial cells, and foci of benign prostatic hyperplasia were manually dissected. Following dissection, bisulfite conversion-specific and methylation-specific PCR (beta actin control) were performed within the laboratory of Dr. David Sidransky as previously described.¹²²⁻¹³⁵

Results specific aim 2:

Estrogen receptor β DNA promoter methylation did not differ when analyzed by treatment group in the three tissue types examined (adenocarcinoma, normal prostate, and benign prostatic hyperplasia). The ratio of estrogen receptor β DNA promoter methylation in adenocarcinoma lesions of the placebo protein treatment group was 30.1, and in the soy protein-containing isoflavonoids group was 66.6. In normal tissue the ratio of estrogen receptor β in the placebo protein treatment group was 16.9 and in the soy protein treatment group it was 26.9. In benign prostatic hyperplasia lesions the ratio of estrogen receptor β promoter methylation was 77.2 in the placebo protein treatment group and 46.0 in the soy protein treatment group. (**Figure 5**)

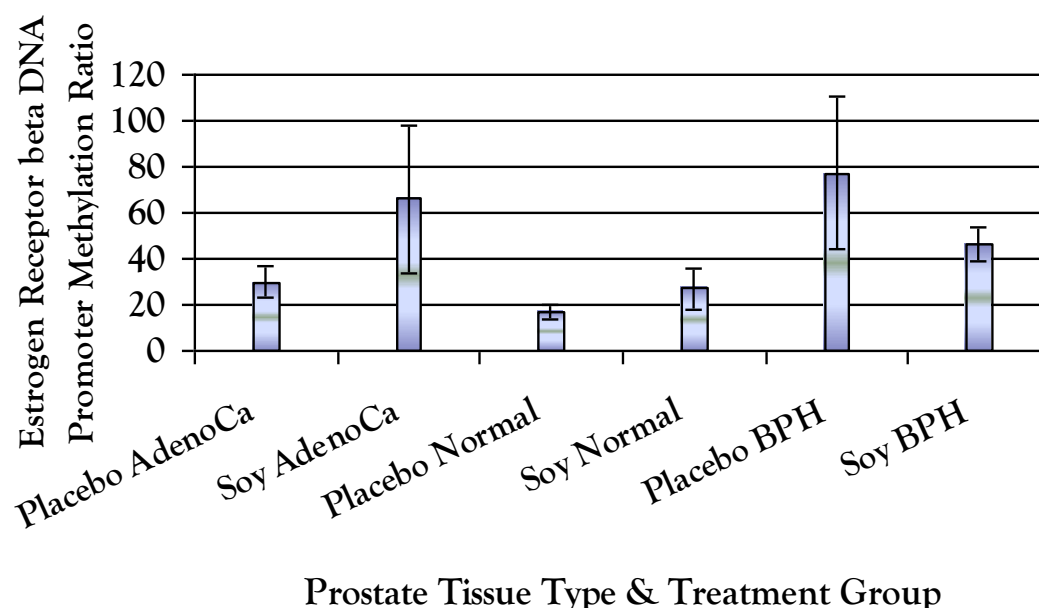


Figure 5. Estrogen Receptor β DNA Promoter Methylation Ratio. There were no significant differences in estrogen receptor β DNA promoter methylation when analyzed by treatment group.

In addition to measuring estrogen receptor β DNA promoter methylation, we measured promoter methylation for five additional genes: the sex hormone receptor estrogen receptor α , the tumor suppressor gene retinoic acid receptor β , the caretaker enzyme glutathione S-transferase pi, the DNA repair enzyme O⁶methylguanine DNA methyltransferase, and the cyclin-dependent kinase inhibitor p16.

We chose to analyze DNA promoter methylation of the estrogen receptor α gene because of its reported estrogen receptor β opposing effects within the prostate. DNA promoter methylation of this gene has been reported to increase dramatically in men with age and increased prostate tumor grade.¹³⁶

The estrogen receptor α DNA promoter methylation ratio in adenocarcinoma lesions was significantly increased in the soy protein treatment group at 113.5 ($P<0.02$) when compared to the placebo protein treatment group. Estrogen receptor α DNA promoter methylation did not significantly differ in normal or benign prostatic hyperplasia (BPH) tissue when analyzed by treatment group. (**Figure 6**)

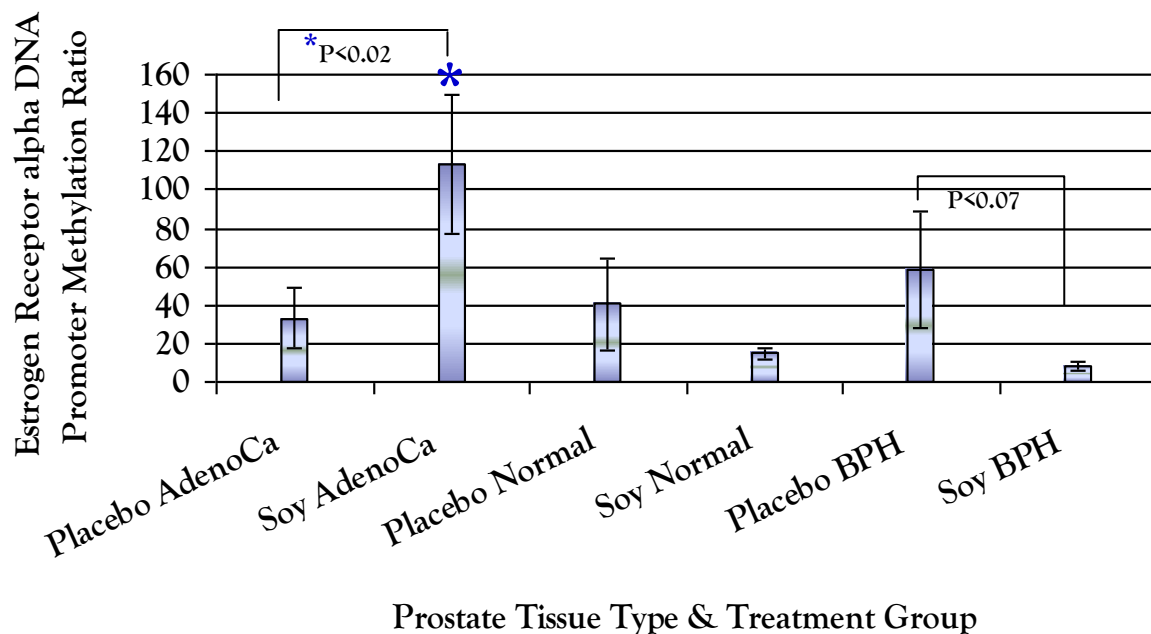


Figure 6. Estrogen Receptor α DNA Promoter Methylation Ratio. Estrogen receptor α promoter methylation was increased in adenocarcinoma ($P<0.02$) in the soy protein-containing isoflavonoids treatment group when compared to the placebo protein treatment group. Estrogen receptor α promoter methylation did not differ in the normal tissue or in benign prostatic hyperplasia when analyzed by treatment group.

Glutathione S-transferase (GSTpi) is a caretaker gene that undergoes marked DNA promoter methylation early in the development of prostate cancer lesions and has been suggested for use as a diagnostic and prognostic marker for the disease.^{127-129,135} Methylation of this gene confirmed proper section of neoplastic prostatic epithelial tissue (versus normal and benign prostatic hyperplasia), and this is the DNA that was utilized for all the DNA promoter methylation analyses. As has been previously reported, DNA promoter methylation of the GSTpi gene was markedly increased in the adenocarcinoma lesions from the prostate glands of the men enrolled in this study and the density of promoter methylation did not differ by treatment group. The

methylation ratio in adenocarcinoma lesions of the placebo protein treatment group measured 1724 and in the soy protein treatment group the methylation ratio measured 1787. The methylation ratio in normal tissue for Glutathione S-transferase measured 27.5 in the placebo protein treatment group and 38.6 in the soy protein treatment group. DNA promoter methylation in benign prostatic hyperplasia lesions was even lower than that of the normal tissue measuring 2.2 in the placebo protein treatment group, and no methylation was detectable in the soy protein treatment group. (**Figure 7**)

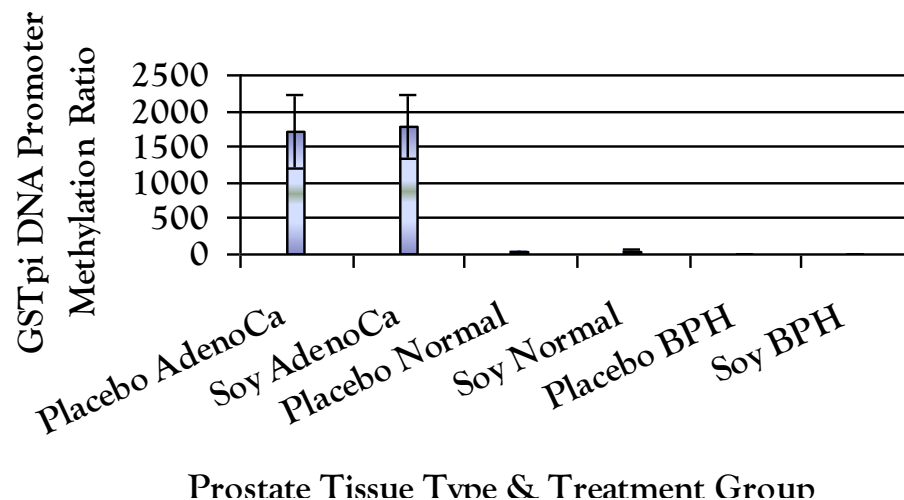


Figure 7. GSTpi DNA Promoter Methylation Ratio. DNA promoter methylation was markedly increased in the prostatic adenocarcinoma lesions in both the placebo protein treatment group and the soy protein treatment group with no significant difference detected when analyzed by treatment group. DNA promoter methylation of GSTpi was almost undetectable in both normal prostate tissue and benign prostatic hyperplasia (BPH).

Retinoic acid receptor β is a tumor suppressor gene that is involved in epithelial cell differentiation. Marked DNA promoter methylation of the retinoic acid receptor β gene has been reported to occur early in prostate cancer development, much like that seen in the glutathione S-transferase promoter,¹³¹ and served to confirm that neoplastic prostatic epithelial tissue (versus normal and benign prostatic hyperplasia) was utilized for the DNA promoter methylation analyses. (**Figure 8**)

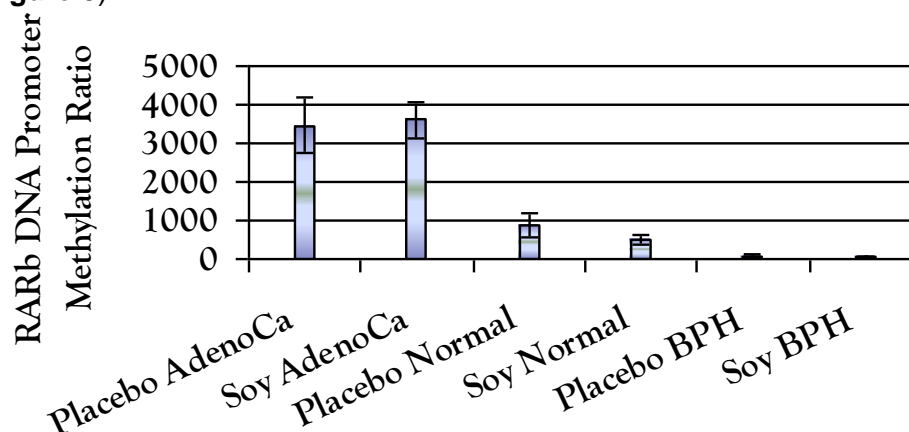


Figure 8. RAR β DNA Promoter Methylation Ratio. DNA promoter methylation was markedly increased in the prostatic adenocarcinoma lesions in both the placebo protein treatment group and the soy protein treatment group with no significant difference detected by treatment group. DNA promoter methylation of RAR β was almost undetectable in both normal prostate tissue and benign prostatic hyperplasia (BPH).

DNA promoter methylation of the DNA repair enzyme O⁶methylguanine DNA methyltransferase and the cyclin dependent kinase inhibitor p16 have been demonstrated by Fang et al. to be reversed by dietary isoflavonoids.¹¹² Neither gene was significantly methylated in the prostate tumors from men in this study when analyzed by treatment group. (**Figure 9**) The cyclin dependent kinase inhibitor p16 exhibited no detectable methylation with the exception of one patient (data not shown).

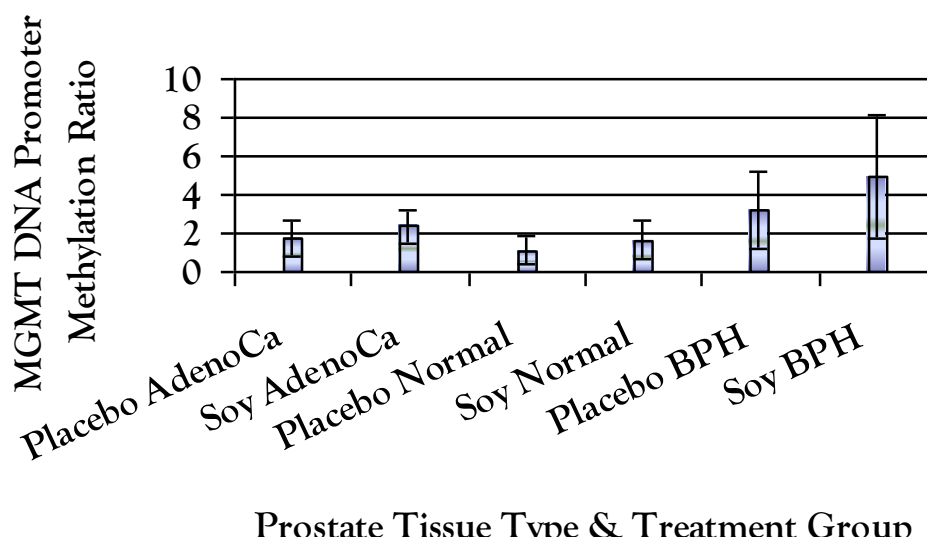


Figure 9. O⁶Methylguanine DNA Methyltransferase DNA Promoter Methylation Ratio. No significant DNA promoter methylation differences were detected by treatment group in the O⁶methylguanine DNA methyltransferase gene in prostatic adenocarcinoma, normal prostate tissue, or benign prostatic hyperplasia.

Aim 3: Correlate intraprostatic soy isoflavonoid concentrations and ER β DNA promoter methylation data with ER β protein expression data and immunohistochemical markers for cell proliferation.

In this aim we examined a) if dietary soy isoflavonoid treatment resulted in increased expression of sex steroid receptor ER β at the protein level, b) if treatment had an antiproliferative effect on neoplastic prostate epithelial cells using proliferation markers Ki67 and PCNA, the cell cycle regulatory marker p27

Stained cells are being counted within foci of adenocarcinoma, areas of prostatic intraepithelial neoplasia, and foci of benign prostatic hyperplasia. Immunohistochemical markers include:

Sex Steroid Markers	ERβ, ERα, AR, PGR
Proliferation Markers	Ki67, PCNA
Cell Cycle Regulatory Markers	p27

Immunohistochemical stains have been performed in Dr. Cline's laboratory on paraffin-embedded tissue with positive and negative controls using antigen retrieval.¹³⁷ Following staining, epithelial then stromal cells are counted at 400x using a 25x25-line grid filtering method as previously reported in breast tissue.¹³⁸ Cells at intersecting cross-hatched lines are the only cells counted. One hundred cells are counted per lesion (benign prostatic hyperplasia,

prostatic intraepithelial neoplasia, and adenocarcinoma). The intensity of avidin-biotin-alkaline phosphatase binding is subjectively assessed as 0 (no staining), 1 (light staining), 2 (moderate staining), or 3 (intense staining).

Results specific aim 3:

Immunohistochemical staining for the sex steroid hormone receptor estrogen receptor β in the epithelium and stroma of prostatic adenocarcinoma (AdCa), normal prostate tissue, benign prostatic hyperplasia (BPH), and prostatic intraepithelial neoplasia (PIN) did not differ by treatment group. (**Figures 10 and 11**) These results follow the DNA promoter methylation ratio results in which there were no significant differences detected when analyzed by treatment group.

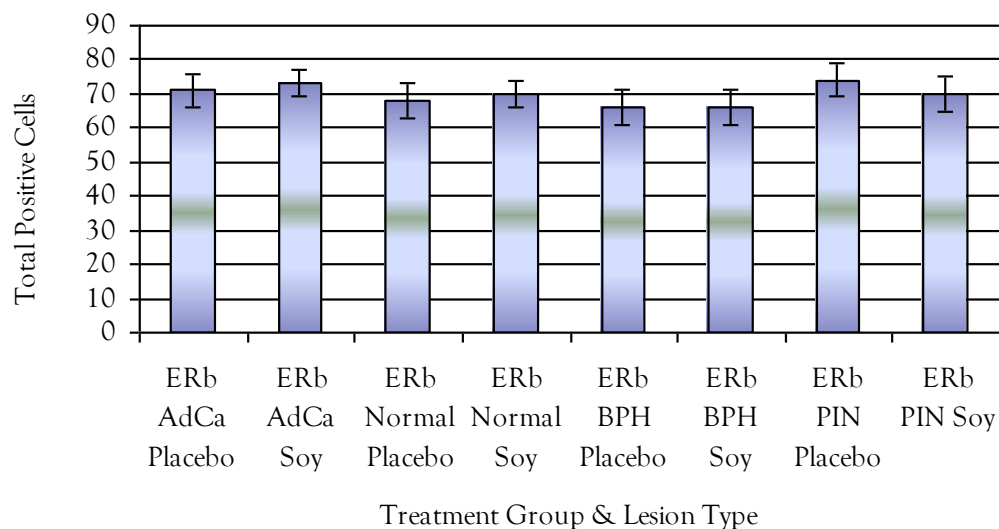


Figure 10. Estrogen Receptor β Immunohistochemical Staining in the Prostate Epithelium. There were no statistically significant differences in estrogen receptor β immunohistochemical staining when examined by treatment group.

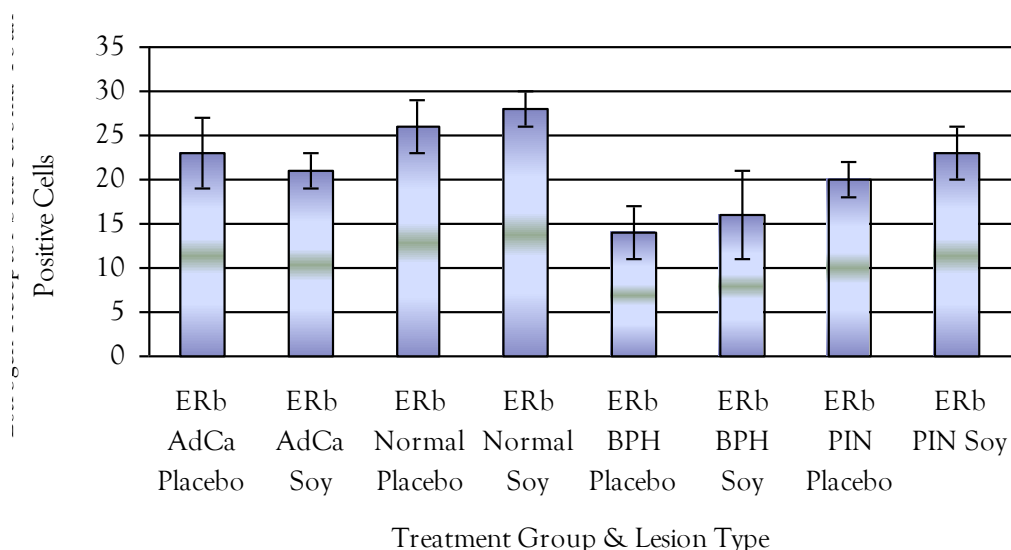


Figure 11. Estrogen Receptor β Immunohistochemical Staining in the Prostate Stroma. There were no statistically significant differences in estrogen receptor β immunohistochemical staining when examined by treatment group.

Immunohistochemical staining for the sex steroid hormone receptor estrogen receptor α in the epithelium and stroma of prostatic adenocarcinoma (AdCa), normal prostate tissue, and benign prostatic hyperplasia (BPH) did not differ by treatment group. (**Figures 12 and 13**) However, estrogen receptor α staining was decreased in the epithelium of adenocarcinoma in men in the soy treatment group at a significance level of 0.06. DNA promoter methylation of the estrogen receptor α gene in prostatic adenocarcinoma of men in the soy protein treatment group was increased, and the decrease in protein expression is consistent with the inverse relationship usually observed between DNA methylation and protein expression, although the protein expression results for estrogen receptor α expression did not reach statistical significance.

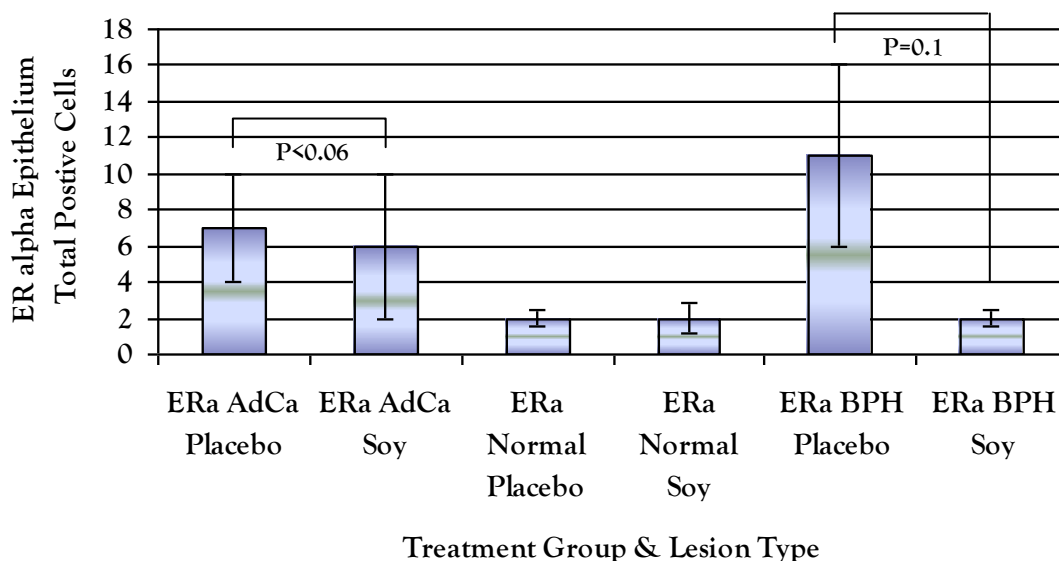


Figure 12. Estrogen Receptor α Immunohistochemical Staining in the Prostate Epithelium. There were no statistically significant differences in ER α immunohistochemical epithelial staining when analyzed by treatment group, although ER α staining in the adenocarcinoma lesions was decreased.

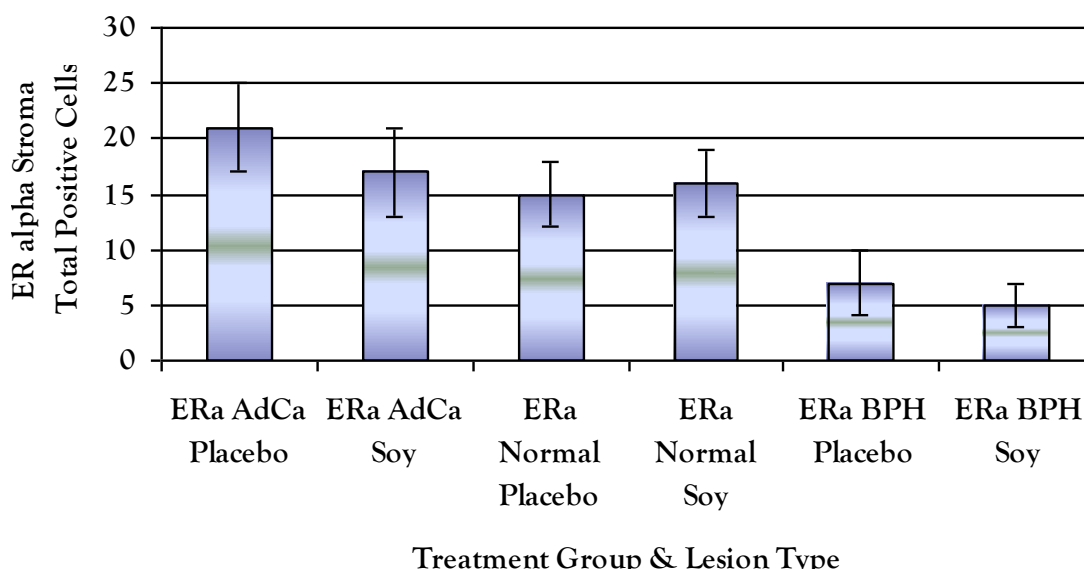


Figure 13. Estrogen Receptor α Immunohistochemical Staining in the Prostate Stroma. There were no statistically significant differences in estrogen receptor α immunohistochemical stromal staining when analyzed by treatment group.

Immunohistochemical staining for the sex steroid hormone receptor androgen receptor in the epithelium and stroma of prostatic adenocarcinoma (AdCa), normal prostate tissue, and benign prostatic hyperplasia (BPH), did not differ by treatment group. (Figures 14 and 15)

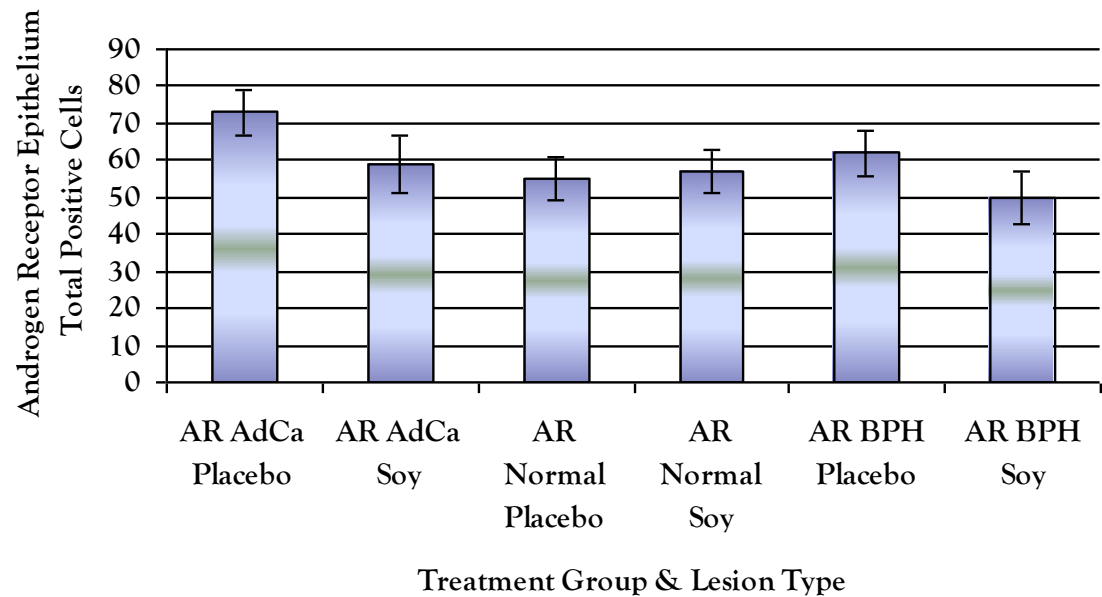


Figure 14. Androgen Receptor Immunohistochemical Staining in the Prostate Epithelium. There were no statistically significant differences in androgen receptor epithelial immunohistochemical staining when analyzed by treatment group.

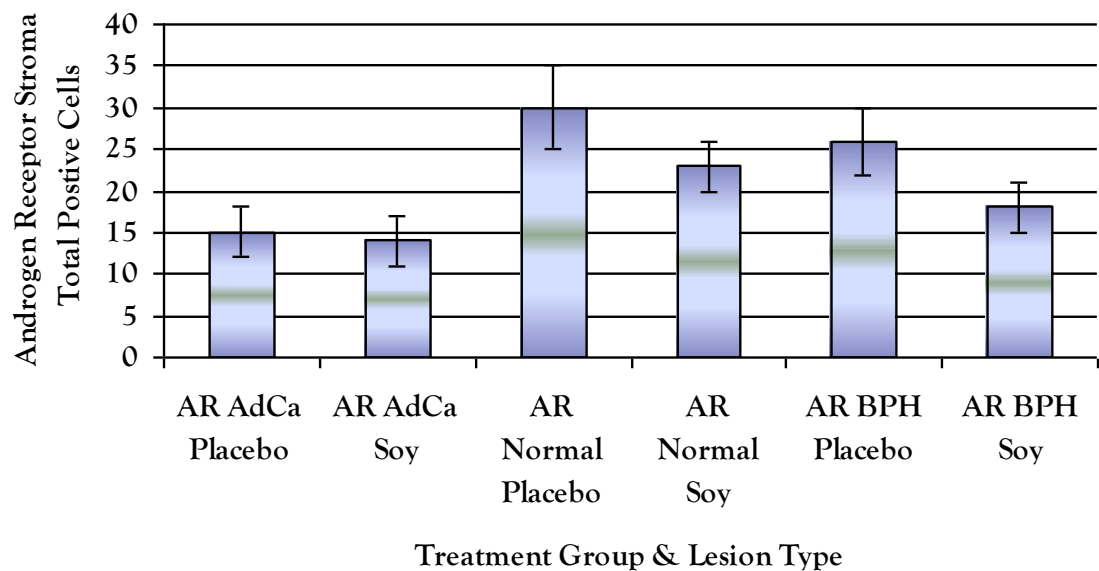


Figure 15. Androgen Receptor Immunohistochemical Staining in the Prostate Stroma. There were no statistically significant differences in androgen receptor immunohistochemical stromal staining when analyzed by treatment group.

Immunohistochemical staining for the proliferation marker proliferating cell nuclear antigen (PCNA) in the epithelium of prostatic adenocarcinoma (AdCa), normal prostate tissue, and benign prostatic hyperplasia (BPH), did not differ by treatment group. (**Figure 16**) PCNA staining was increased at the $P<0.05$ level in the prostate stroma of the soy protein treated men. (**Figure 17**)

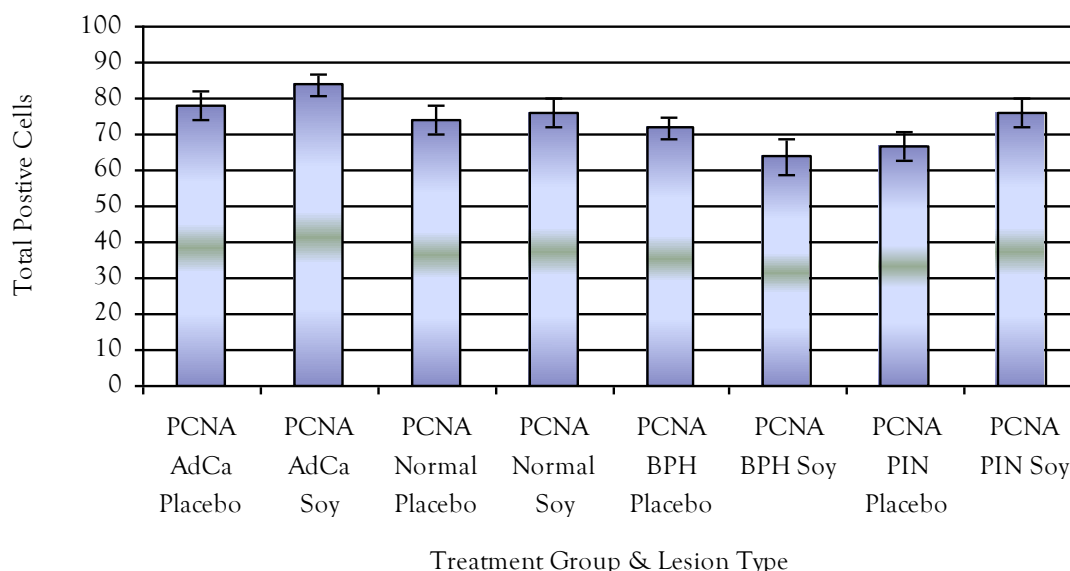


Figure 16. Proliferating Cell Nuclear Antigen (PCNA) Immunohistochemical Staining in the Prostate Epithelium. There were no statistically significant differences in PCNA immunohistochemical staining when analyzed by treatment group.

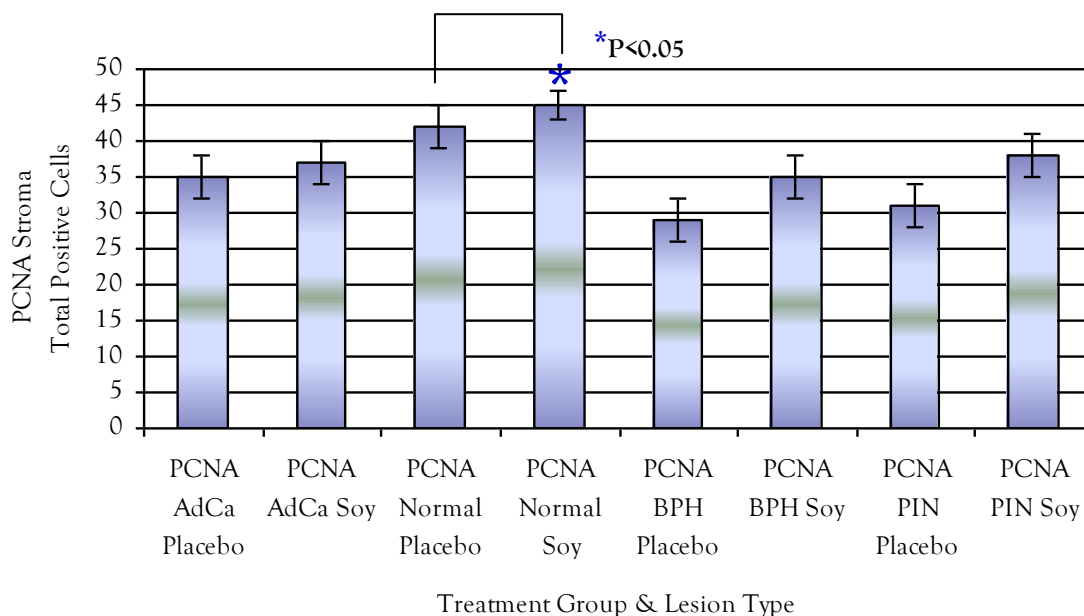


Figure 17. Proliferating Cell Nuclear Antigen (PCNA) Immunohistochemical Staining in the Prostate Stroma. PCNA staining was increased ($P<0.05$) in the normal tissue of the soy protein treated men.

Immunohistochemical staining for the proliferation marker Ki67 in the epithelium and stroma of prostatic adenocarcinoma (AdCa), normal prostate tissue, and benign prostatic hyperplasia (BPH), and prostatic intraepithelial neoplasia (PIN) did not differ when analyzed by treatment group. (**Figures 18 and 19**) Ki67 staining is significantly increased in the adenocarcinoma lesions versus normal tissue or benign prostatic hyperplasia lesions although we have only analyzed the data by treatment group thus far.

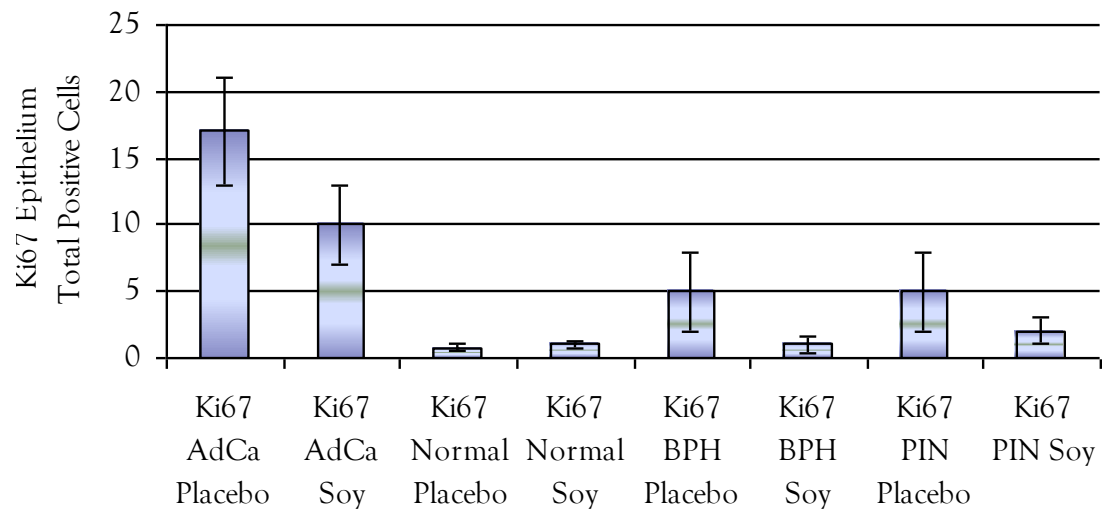


Figure 18. Ki67 Immunohistochemical Staining in the Prostate Epithelium. Ki67 epithelial staining did not differ when analyzed by treatment group.

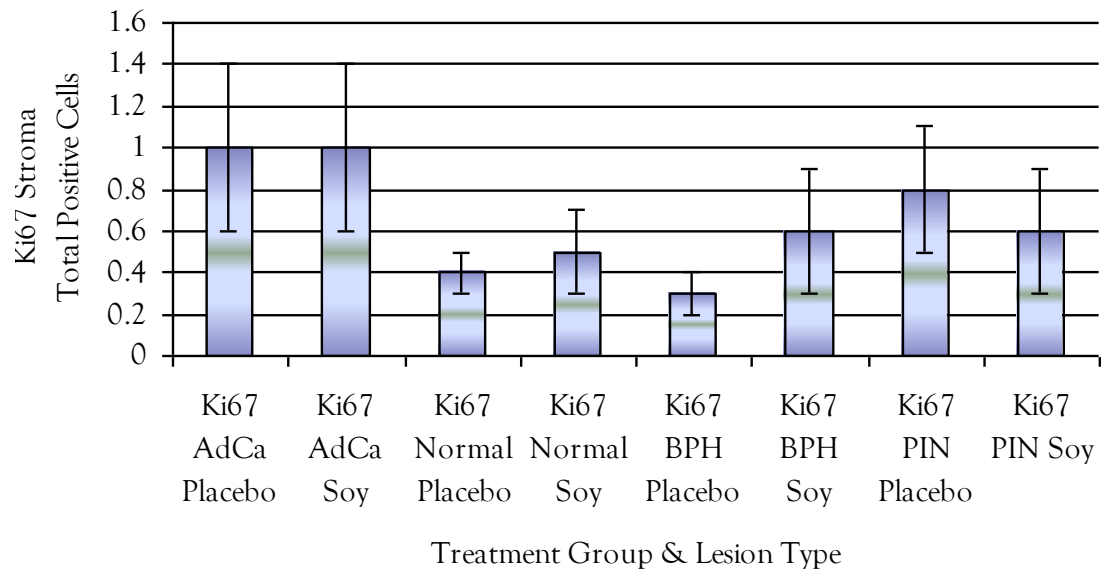


Figure 19. Ki67 Immunohistochemical Staining in the Prostate Stroma. Ki67 stromal staining did not differ when analyzed by treatment group.

Key Research Accomplishments:

- Completed intraprostatic isoflavonoid concentration measurements from frozen prostate tissue with the assistance of our collaborator Dr. Adrian Franke at the Cancer Research Center of Hawaii, and completed data analysis/comparisons to serum isoflavonoid concentrations by dietary protein treatment group.
- Properly selected and isolated prostatic tissue from paraffin sections of tissue including adenocarcinoma, normal prostate tissue, and benign prostatic hyperplasia, and isolated the DNA from these tissues for DNA promoter methylation analysis.
- Traveled to Johns Hopkins University and completed the estrogen receptor β , estrogen receptor α , glutathione S-transferase pi, retinoic acid receptor β , O⁶methylguanine DNA methyltransferase, and p16 DNA promoter methylation analyses, and analyzed this data by dietary protein treatment group with the generous assistance of Dr. David Sidransky's laboratory.
- Completed immunohistochemical staining and quantification of staining in the epithelium and stroma for the sex steroid receptor markers estrogen receptor β , estrogen receptor α , androgen receptor, and the proliferation markers proliferating nuclear cell antigen (PCNA) and Ki67 with data analysis by dietary protein treatment group.

Reportable Outcomes:

- Four-week dietary treatment with soy protein containing isoflavonoids at a dose of 100mg/day did not result in an increase in isoflavonoid concentrations within the prostate gland tissue when compared to concentrations measured in the serum.
- Four-week dietary treatment with soy protein containing isoflavonoids at a dose of 100mg/day did not alter estrogen receptor β DNA promoter methylation density or estrogen receptor β protein expression within the epithelium or stroma of prostate adenocarcinoma in men with an average Gleason score of 6, normal prostate tissue, and benign prostatic hyperplasia.
- Four-week dietary treatment with soy protein containing isoflavonoids at a dose of 100mg/day resulted in an increase in DNA promoter methylation of the estrogen receptor α gene in the prostate adenocarcinoma tissue of men with an average Gleason score of 6 at the 0.02 significance level when compared to the placebo (milk) protein treatment group.
- Four-week dietary treatment with soy protein containing isoflavonoids at a dose of 100mg/day did not alter glutathione S-transferase pi DNA promoter methylation density in adenocarcinoma lesions of the prostate gland when analyzed by treatment group with an average Gleason score of 6 though DNA promoter methylation was markedly increased in adenocarcinoma lesions when compared to normal prostate tissue and benign prostatic hyperplasia lesions as has been previously reported.

- Four-week dietary treatment with soy protein containing isoflavonoids at a dose of 100mg/day did not alter retinoic acid receptor β DNA promoter methylation density in adenocarcinoma lesions of the prostate gland with an average Gleason score of 6 though DNA promoter methylation was markedly increased in adenocarcinoma lesions when compared to normal prostate tissue and benign prostatic hyperplasia as has been previously reported.
- No significant DNA promoter methylation was detected in either dietary protein treatment group in prostate adenocarcinoma lesions with an average Gleason grade of 6 of the DNA repair enzyme O⁶methylguanine DNA methyltransferase gene.
- No significant DNA promoter methylation was detected in either dietary protein treatment group in prostate adenocarcinoma lesions with an average Gleason score of 6 of the cyclin dependent kinase inhibitor p16 gene.
- Four-week dietary treatment with soy protein containing isoflavonoids at a dose of 100mg/day resulted in an increase in the proliferation marker proliferating nuclear cell antigen (PCNA) at the 0.05 significance level in the stroma of normal prostate tissue when compared to placebo (milk) protein but PCNA expression did not differ in the epithelium or stroma of neoplastic prostate tissue or benign prostatic hyperplasia.
- Four-week dietary treatment with soy protein containing isoflavonoids at a dose of 100mg/day resulted in no detectable difference in expression of the proliferation marker Ki67 in the epithelium or stroma of neoplastic prostate tissue, normal prostate tissue, or benign prostatic hyperplasia.

Conclusions:

In this study we hypothesized that:

Estrogen receptor β (ER β) epigenetic silencing through DNA promoter methylation in neoplastic prostatic epithelial cells can be reversed by dietary soy isoflavonoids when given at physiologically achievable levels, as shown by a positive correlation between serum and/or intraprostatic isoflavonoid concentrations and ER β expression and an inverse correlation with neoplastic cell proliferation

Our results do not demonstrate a decrease in estrogen receptor β DNA promoter methylation or protein expression after four weeks of dietary soy protein treatment delivering a dose of 100mg of isoflavonoids daily. Decreases in estrogen receptor β protein expression with associated increases in DNA promoter methylation have been reported most often in tumors of Gleason grade 4 or higher while the average Gleason grade in our study was 3.^{91,102} This may explain our finding of no significant increase in DNA promoter methylation of the estrogen receptor β gene in the neoplastic prostate epithelium of our samples.

Although we demonstrated no change in estrogen receptor β DNA promoter methylation density within the neoplastic prostate epithelial tissue after 4 weeks of dietary isoflavonoid treatment at a dose of 100mg daily, estrogen receptor α , a receptor that is normally expressed predominantly in the prostate tissue stroma and not in the epithelium exhibited a statistically significant increase in methylation after the 4-week dietary soy isoflavonoid treatment.

An increase in estrogen receptor α DNA methylation within the epithelium was associated with a decrease in estrogen receptor α protein expression within the epithelium, although this decrease in protein expression did not reach statistical significance. This change in estrogen receptor α protein expression with soy isoflavonoid treatment would restore the prostate epithelium to a more normal state as estrogen receptor α is normally expressed predominantly in the prostate tissue stroma. This is evidenced in a study performed by Lau et al. in 2000 where estrogen receptor subtype expression was analyzed in normal and malignant human prostate epithelial cells.¹⁰⁷ Normal prostate epithelial cells expressed exclusively estrogen receptor β transcripts and no estrogen receptor α . Estrogen receptor α transcripts were detected in the epithelium of the malignant prostate cancer cell line PC-3 and in an immortalized prostate cancer cell line (PrEC) and in epithelial cells isolated from benign prostatic hyperplasia lesions (**Figure 20**). Estrogen receptor α over-expression in prostate adenocarcinoma also has been reported with increasing prostate tumor grade.¹⁰¹ In our study estrogen receptor α promoter methylation was decreased with a dietary soy protein isoflavonoid supplementation of just four weeks with a concomitant decrease in estrogen receptor α expression. This is a novel and exciting finding.

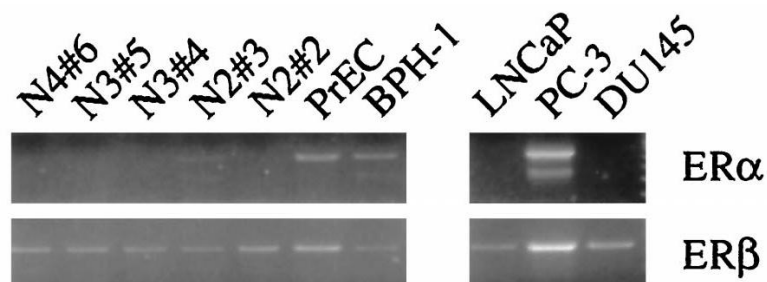


Figure 20. Estrogen receptor α and β expression in normal prostate epithelial cells versus immortalized epithelial cell lines and benign prostatic hyperplasia. Estrogen receptor α transcripts are expressed only in immortalized or malignant prostate cancer cell lines and the epithelium of benign prostatic hyperplasia. Lau et al. Cancer Research 2000.

The cell cycle repair enzyme O⁶methylguanine DNA methyltransferase was not methylated significantly in the prostate gland samples from this study. Although promoter methylation of this enzyme has been reported in some studies up to 55%^{134,139} and methylation has been reported to be reversed by soy isoflavonoid treatment *in vitro*¹¹², most publications exploring O⁶methylguanine DNA methyltransferase promoter methylation are consistent with our data and report little to no DNA promoter methylation of the O⁶methylguanine DNA methyltransferase gene in prostate cancer.^{126,135,140}

Although the cyclin dependent kinase inhibitor p16 has been shown to be inactivated by DNA promoter methylation in prostate adenocarcinomas,^{134,141} we detected no DNA promoter methylation of the cyclin dependent kinase inhibitor p16 gene in the prostate samples from this study with the exception of one patient. This is consistent with reports that the protein expression levels of this cyclin dependent kinase inhibitor increase with increasing prostate cancer grade.¹⁴²⁻¹⁴⁴

The increase in immunohistochemical staining of proliferating nuclear cell antigen (PCNA) in the stroma of normal prostate tissue at a significance level of 0.05 indicates that soy isoflavonoid treatment resulted in increased proliferation of these stromal cells, although immunohistochemical staining for our second proliferation marker, Ki67 was not increased in the stroma of normal tissue in the men in the soy isoflavonoid treatment group when compared to the placebo protein treatment group. Soy consumption has been associated with fewer or smaller benign prostatic hyperplasia lesions and less prostatic inflammation in rodent and cell culture models.^{145,146} Higher genistein levels in the prostate tissue of men have been associated

with less benign prostatic hyperplasia.¹⁴⁷ However, estrogens have been implicated in the pathogenesis of benign prostatic hyperplasia and soy isoflavonoids do bind to the estrogen receptor.¹⁴⁸ Quantification of the estrogen responsive gene, progesterone receptor, which is upregulated in tissues following treatment estrogen or estrogen-like compounds,¹⁴⁹ is pending in these tissues, and progesterone receptor expression will indicate if the soy isoflavonoid treatment had an estrogenic effect in the stroma of normal prostate tissue.

We found no increase in prostate tissue isoflavonoids when compared to concentrations measured in the serum. This finding is in contrast to studies by Morton et al., Hedlund et al., Ranninkko et al., and Gardener et al.^{116,117,119,150} who have found 4- to 23-fold increases in prostate tissue isoflavonoid concentrations in men consuming soy isoflavonoids when compared to serum concentrations. The half-life of soy isoflavonoids is approximately 3 to 6 hours in the serum of normal healthy individuals.¹⁵¹ The last dose of soy isoflavonoids taken by the men in this study was the night prior to their prostatectomy surgery. This would mean a minimum of 8 to 12 hours between the last dose of soy isoflavonoid supplement consumed and fresh prostate tissue collection for snap freezing. We speculate that the low levels of isoflavonoids in the prostate tissue of the men in this study was due to this extended time period between last isoflavonoid dose and tissue collection, and it provides some insight into the prostate tissue half-life of isoflavonoids, which based on our results, is likely not longer than that in the serum.

Quantification of immunohistochemical stains for progesterone receptor and the cell cycle regulatory molecule p27 are ongoing. This study will further what is currently known about the role of diet in shaping our epigenome and specifically how soy protein containing isoflavonoids may influence the progression of prostate cancer. Our understanding of estrogen and estrogen-like compounds in the development of prostate disease is just beginning.^{90,92,152}

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